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(54) Title: PHARMACEUTICALLY EFFECTIVE COMPOUNDS AND THEIR USE

(57) Abstract: According to the invention we provide use of panaxatriol for the treatment of conditions requiring stimulation of angiogenesis, and use of panaxatriol in the manufacture of medicaments for such treatment. Preferaby the panaxatriol is the naturally-occurrig ginsenoside Rg1.

Pharmaceutically effective compounds and their use

This invention relates to compounds which we have found to have angiogenic effects, in particular the ginseng component Rg_1 and its derivatives, and other angiogenic panaxatriol ginsenosides, and their use in therapeutic applications for which an angiogenic response is valuable. The invention also relates to compositions containing Rg_1 and other panaxatriol ginsenosides and their production.

The root of *Panax ginseng* has been well known as a general "tonic" for many centuries. It is used for its anti-stress effects and for toning up the nervous system, and has been suggested for a variety of other purposes, including treatment of atherosclerosis, cerebrovascular diseases, liver dysfunction, post-menopausal disorders and hypertension.

Attele *et al* in Biochem. Pharmacol. Vol. 58, pp 1685-1693, 1999, review the various pharmacological effects which have been reported for ginseng.

Morisaki *et al* in Br. J. Pharmacol. (1995) 115, 1188-1193 surmise that saponin of ginseng *Radix rubra* influences angiogenesis. They state that orally administered ginseng has been reported to stimulate the repair of intractable skin ulcers in some patients. Morisaki *et al* suggest a mechanism of tube formation by saponin modifying the balance of protease/protease inhibitor secretion from HUVEC and enhancing the migration of HUVEC.

Kanzaki *et al* in Br. J. Pharmacol. (1998) 125,255-262, disclose some effects of saponin on wound healing. They report the Morisaki *et al* suggestion that saponin stimulates wound healing by the suggested mechanism above, but carry out further testing to clarify this. They surmise that saponin stimulates fibronectin synthesis through changes of TGF-β receptor expressions in fibroblasts.

Although these authors have surmised possible effects on wound healing of saponin of ginseng the mechanism of

wound healing is so complex and so many factors are involved that the situation is far from certain.

White *et al* in Pharmacotherapy July 2001: 21(7): 773-7, "An Evaluation of the Hemostatic Effects of Hydrophilic, Alcohol and Lipophilic Extracts of Notoginseng" disclose that notoginseng extracts can have hemostatic effects. The same authors discuss use of notoginseng extracts for hemostatic effects in J. Clin. Pharmacol. 2000 Oct.: 40(10): 1150-3.

Separately, Sato *et al* in Biol. Pharm. Bull. 1994 May; 17(5):635-9, report that one ginsenoside, Rb_2, inhibited tumour growth and showed an anti-angiogenic effect.

Mochizuki *et al* in Bio. Pharm. Bull. 1995; Sep; 17 (9): 1197-202 disclose that saponin preparations 20(R)-and 20(S)-ginsenoside-Rg_3 inhibit the lung metastasis of tumour cells and suggest that this mechanism is related to inhibition of the adhesion and invasion of tumour cells and to anti-angiogenic activity.

Therefore, the effects of ginseng on angiogenesis are at present uncertain. As can be seen from above, it has been suggested both that it exhibits angiogenic effects and that it exhibits anti-angiogenic effects.

A known component of ginseng is the panaxatriol ginsenoside Rg_1. Rg_1 has a four trans-ring steroid skeleton and is a panaxatriol (see Figure 1a). Lee *et al* (1997) Mol. Cell. Endocrinol., 133, 135-140 report that Rg_1 is a functional ligand of the glucocorticoid receptor. Thus this would suggest that Rg_1 mediates impairment of wound healing.

We have now found surprisingly that, contrary to what would be expected from the suggestions by Lee *et al*, the ginsenoside Rg_1 exhibits angiogenic effects.

Thus according to a first aspect of the invention we provide a method of stimulating angiogenesis in a human or other animal subject by the use of a panaxatriol, preferably Rg_1. The invention also provides the use of a

panaxatriol, preferably Rg_1, in the manufacture of a medicament for treatment of a human or other subject by stimulation of angiogenesis.

As discussed above, this conclusion is surprising, firstly in view of the general uncertainty about the angiogenic effects of ginseng as a whole, with Kanzaki *et al* and Morisaki *et al* suggesting that ginseng extract may have angiogenic effects but Sato *et al* and Mochizuki *et al* suggesting that certain components (and thus ginseng itself) have an anti-angiogenic effect. It is moreover especially surprising in view of the fact that of all the components of ginseng Rg_1 would be expected to have effects which impair wound healing (Lee *et al*).

Rg_1 is in the panaxatriol or 20(S)-protopanaxatriol group of ginsenosides (Gillis, Biochem. Pharmacol., vol. 54, pp. 1-8, 1997. It should be noted that we believe the formula shown there to be incorrect. The correct formulae are given in Figure 1. Specifically, it is a 20(s)-protopanaxatriol, having a chiral C_{20}. We believe that other panaxatriols also exhibit equivalent angiogenic effects. Discussion below which concerns Rg_1 but is equally applicable to other angiogenic panaxatriols and their derivatives. We note also that Rb_2 and 20 (R) and 20 (S) - ginsenoside Rg_3 are all panaxadiols, although the authors of the papers on these discussed above do not discuss this fact.

Rg_1 has a known structure, described by Shibata *et al*, (1985) "Chemistry and Pharmacology of Panax", in Economic and Medical Plant Research, Academic Press, New York, Vol. 1, pp. 217-284. The structure of Rg_1 is given in Figure 1(a). In the natural form the rings are in the trans configuration.

Panaxatriols have the structural formula in Figure 1 (b). They are based on the triterpene dammarane structure. Some panaxatriols are naturally-occurring ginsenosides.

In the formula 1(b) each of R_1, R_2, R_3 and R_4 may independently be H or any organic group having up to 30 carbon atoms. They may be the same or different. In particular they may be sugar-containing groups. The sugar groups may be 5-ring sugars or 6-ring sugars and may for instance be selected from glucose, maltose, fructose, xylose, rhamnose and arabinose. They may alternatively be alkyl, alkenyl or alkynyl so that R_1O-, R_2O-, R_3O- and/or R_4O- are ethers. Alternatively they may be acyl groups so that the R_1O-, R_2O-, R_3O- and/or R_4O- groups are esters. For instance R_1, R_2, R_3 and R_4 may be fatty acyl, saturated or unsaturated. Preferably R_1 is H. R_4 is preferably H. Preferably R_2 and R_3 are sugar-containing groups. Preferably they contain only sugar moieties but they may be derivatives of sugars.

Preferably each of R_2 and R_3 has not more than 24, preferably not more than 18 and particularly preferably not more than 12 carbon atoms. Preferably R_3 has not more than 18 carbon atoms, more preferably not more than 12 carbon atoms, most preferably not more than 6 carbon atoms.

Compounds of the Formula 1(b) may have any stereochemical structure. Preferably the stereochemical structure of the 4-ring skeleton is trans-trans-trans as in naturally occurring Rg_1.

Within any structure for which the steroid ring stereo chemistry is defined, the stereochemistry at C20 may be R or S. Thus any defined skeleton stereostructure can produce two enantiomers. The S-configuration is preferred. Panaxatriols may be used in the form of a single enantiomer or a non-racemic mixture of enantiomers or as the racemate. Panaxatriols having the structural formula of a naturally-occurring ginsenoside preferably also have the stereochemistry of the naturally occurring ginsenoside.

Mixtures of panaxatriols may be used.

The compounds used in the invention differ from panaxadiols in that in the latter the group OR_2 is instead H. In the invention panaxatriol is used without

panaxadiol. Thus the use of non-purified ginseng or saponin of ginseng is excluded. Preferably we use only one or two active constituents, each of which is a panaxatriol. Thus in the invention we use the surprising realisation that the panaxatriol constituents of ginseng, in particular Rg_1, are those which are responsible for angiogenic activity.

The structures of the naturally-occurring ginsenosides Re, Rf, Rg_2 and Rh_1 are known. In these naturally-occurring panaxatriols the groups R_1 and R_4 are H. In Rg_1 the group R_2 is -Glc-. In Re it is -Glc^2-Rha, in Rf it is -Glc^2-Glc and in Rg_2 it is -Glc^2-Rha. In Re and Rg_1 the group R_3 is Glc and in Rf and Rg_2 the group R_3 is H. Rg_2 exists naturally in the 20R form. Rg_1, Re and Rf exist naturally in the 20S form. In Rh_1 the group R_2 is -Glc and the group R_3 is H. Rh_1 exists naturally in the 20S and 20R forms. These naturally-occurring panaxatriols have the same steroid ring stereochemistry as Rg_1 and may be obtained from the leaves and seeds of *Panax ginseng*.

Other naturally-occurring panaxatriols which may be used include compounds commonly described as notoginsenosides. These are found naturally in the leaves and seeds of the species *Panax notoginseng*. The notoginsenoside-R1 has the formula 1(b) in which R_1 is H, R_2 is Glc^2-Xyl, R_3 is Glc and R_4 is H. Notoginsenoside-R2 has the formula 1(b) in which R_2 is Glc^2-Xyl and R_3 is H. Notoginsenoside-R3 has the formula 1(b) in which R_2 is Glc and R_3 is Glc^6-Glc. Notoginsenoside-R6 has the formula 1(b) in which R_2 is Glc and R_3 is Glc^6-Glc (α configuration). The steroid ring stereochemistry is the same in these naturally-occurring notoginsenosides as in the ginsenoside Rg_1. They exist naturally in the 20S form.

Naturally-occurring panaxatriols for use in the invention may be obtained from various ginseng species. These include *Panax ginseng* (often described as Oriental ginseng), *P. quinquefoliens* (often described as American ginseng), *P.notoginseng* and *P.japonicus* C.A.Mey (often

described as Japanese ginseng). Preferably they are obtained from *P.ginseng* or *P.notoginseng*, preferably *P.ginseng*.

All of the naturally-occurring ginsenosides have the same stereostructure of the four-ring steroid skeleton. The structure is trans-trans-trans.

Purification of Rg_1 and other naturally-occurring panaxatriols from ginseng may be achieved by known methods. Derivatives of the formula in Figure 1 (b) may be used. These will have equivalent functionality. That is, any derivatisation does not significantly reduce the angiogenic effect. Generally functionally equivalent derivatives have the same or a greater angiogenic effect than Rg_1. If it is a lesser effect it is generally not less than 70%, usually not less than 80%, of the effect of Rg_1.

Suitable panaxatriols of the formula in Figure 1(b) may be derived using methodologies such as quantitative structure activity relationships (QSAR) and comparative molecular field analysis (CoMFA) so that molecules having equivalent or increased effectiveness over naturally-occurring ginsenosides may be devised using the naturally-occurring ginsenosides as starting points. This methodology is discussed for instance by Richon and Young, in "An Introduction to QSAR Methodology", Network Science, 2000. Software packages such as QSAR with CoMFA, available from the company Tripos Inc may be used, but other products are available.

It is also possible to use such techniques to derive compounds not of the Formula 1(b) but which show equivalent functionality to Rg_1 or other panaxatriol. In particular, the CoMFA methodology, a three-dimensional QSAR technique, is useful. For instance, techniques are known for defining the steric and electronic features of a compound necessary to ensure optimal supramolecular interactions with a specific biological target structure. This ensemble of steric and electronic features is known as a pharmacophore. Similar techniques may be used to define other molecules

having the steric and electronic features provided by Rg_1 or other naturally-occurring ginsenoside.

In the treatment methods Rg_1 or other panaxatriol may be applied to the subject in any convenient manner. References below to Rg_1 are also applicable to other panaxatriols.

Preferred vehicles include liposomes, microsomes or microsponges within which the Rg_1 may be contained and which may be injected for instance intravenously, intramuscularly or subcutaneously. Cationic liposomes and stealth liposomes with or without antibody coating may be used.

Other suitable vehicles include implants, such as collagen implants, in a suitable form for application to the subject. Implants may be made of natural (eg collagen, fibrin or gelatin), synthetic (eg methylcellulose, ELVAX, tetradecacyclodextran) or semi-synthetic materials. They may be applied to a wound or ulcer for which healing is required. Other application forms include topical application, eg as a cream, gel, transdermal patch or time-controlled drug release system, eg polymeric drug release system. The Rg1 may be used impregnated in materials which are introduced to the body of the subject for other reasons, eg sutures (eg made of catgut) or vascular grafts.

Applications for which the angiogenic activity of Rg_1 is beneficial include wound healing and ulcer healing. Other indications include non-union fractures, myocardial infarction (for subjects having elevated risk of or having experienced myocardial infarction), stroke (for subjects having elevated risk of or having experienced ischaemic stroke), hair loss, gangrene, transplantation (eg after tissue transplantation) and in certain neurological and ophthalmological conditions where enhanced neovascularisation is deemed to be beneficial to human or other animal subjects. The angiogenic activity of Rg1 may also be applied in transplantation, for instance impregnated in skin grafts or vascular grafts.

The subject treated may be any animal, preferably a mammal, and is usually a human.

The Rg_1 may be used in the form of any suitable pharmaceutical composition, generally provided by purifying Rg_1 from ginseng and combining it with a suitable pharmaceutical excipient. Pharmaceutical excipients can be chosen on the basis of general knowledge.

The pharmaceutical composition may be in a form suitable for oral administration, for instance tablets, capsules or a gel. Alternatively, it may be an aerosol formulation. It can thus be sprayed onto any biological surface such as the skin or internal organs. A particularly suitable composition comprises Rg_1 in artificial or tissue-engineered skin or in slow drug release skin patches. Others include the forms such as sutures, vascular grafts and skin grafts discussed above. Compositions may also be in a form suitable for use in conjunction with gene-derived therapy.

Dosages may be chosen by those skilled in the art using known methods. The dosage is chosen so that the panaxatriol is administered in an amount effective to stimulate angiogenesis in the subject. Suitable dosages may be from 25 ng to 500 μg panaxatriol per kg per day, preferably from 5 μg to 100 μg panaxatriol per kg per day. Treatment may be carried out for any period of time and may be over a period of months or even years. However, generally treatments will be carried out for from 1 to 60 days, preferably 1 to 30 days.

The results below show that Rg_1 produces an angiogenic response both in the *in vivo* mouse sponge granuloma model discussed below and in the Lauder *et al in vitro* HUVEC model also discussed below.

As can be seen from the results below, we find that Rg_1 upregulates certain genes. Thus we conclude that these genes are implicated in angiogenesis and according to a further aspect of the invention we provide a method of testing a target compound for angiogenic activity

comprising performing an assay to determine whether the target compound upregulates any of the genes IQGAP1, RhoB, RhoC, Rho, AbetaPP, Rac-alpha, Rac1, PRAD1, HSP90, TGFbeta3, CALM2, LAMA4, LAMB1, LAMB2. Preferably the assay determines whether the target compound upregulates any of the genes IQGAP1, RhoB, RhoC, Rho, AbetaPP, Rac-alpha, Rac1, HSP90, CALM2. The assay is *in vitro*. It is generally performed by treating HUVECs with the target compound for at least two hours, preferably from 6 to 48 hours, often from 20 to 28 hours, and testing for upregulation. This may be done for instance by extracting RNA from the cells and testing for hybridisation with the defined genes. Generally the genes are provided as polynucleotides on an array. Other methods for ananlysing and detecting changes in RNA level/gene expression may be used by those skilled in the art. They include for instance reverse transcriptase polymerase chain reaction. Other detection methods such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) can be used.

We believe that the mechanism by which Rg_1 induces angiogenesis could be mediated by nitric oxide (NO).

Recently, NO has been indicated as a signalling molecule in angiogenesis although its precise role is controversial. In the examples below we demonstrate our findings which suggest that the angiogenic effects of Rg_1 could be mediated through the activation, by means of an increased expression of eNOS leading to NO synthesis.

Gillis in "Panax Ginseng Pharmacology: A Nitric Oxide Link?", Biochem. Pharmacol., Vol. 54, 1-8, 1997, suggested that the activity of ginseng could be linked to enhanced nitric oxide synthesis in the endothelium. It is suggested that Rg_1 enhances the conversion of L-arginine to L-citrulline and that bradykinin also increases this conversion and that bradykinin is known to activate eNOS.

Kang *et al* in Life Sci. 1995, 56:1577-86, "Ginsenosides of the Protopanaxatriol Group Cause Endothelium-Dependent Relaxation in the Rat Aorta" disclose

that protopanaxatriol ginsensosides Rg_1 and Re cause endothelium dependent relaxation which is inhibited by an inhibitor of nitric oxide synthase. The authors conclude that protopanaxatriols ginsenosides enhance the release of nitric oxide from endothelial cells.

Chen in Clin. Exp. Pharmacol. Physiol. 1996, Org. 23:728, 32, "Cardiovascular Protection by Ginsenosides and Their Nitric Oxide Releasing Action", teaches that Rg_1 relaxed pulmonary vessels, an effect which was eliminated by nitro-l-arginine, an inhibitor of nitric oxide synthase.

Fan *et al*, in Am. J. Chin. Med. 1995, 23:279-87, "Enhancement of nitric oxide production from activated macrophages by a purified form of ginsenoside (Rg_1)" disclose that Rg_1 enhanced the production of nitric oxide from IFN-gamma activated macrophages or RAW cells and from macrophages cocultured with non-adherent spleen cells stimulated by Con A, LPS, or anti-CD3.

However, none of these references address the mechanism by which Rg_1 might be increasing nitric oxide production, in particular its precise influence on the activity of eNOS.

We have found that it is the activity of Rg_1 and other panaxatriols in increasing expression of the eNOS enzyme which leads to increased nitric oxide synthesis. We believe that the eNOS enzyme is increased in activity, but, crucially, the Rg_1 or panaxatriol causes larger amounts of eNOS to be present.

We find that this effect of Rg_1 is useful for other indications (other than angiogenesis) in which increased concentration of NO is beneficial.

Nitric oxide is known to be a messenger molecule having a variety of roles in the cardiovascular, neurologic and immune systems. The enzyme NO synthase (NOS) produces NO from the guanidino nitrogen of L-arginine. Three isoenzymes of NOS have been identified in mammals. The isoenzyme with which this specification is particularly concerned, eNOS, is expressed in endothelial cells and is

calcium-dependent. nNOS, expressed in neurons, is also calcium-dependent. The third type, iNOS, is calcium-independent and is expressed by macrophages and other cells after induction with cytokines.

The recent publications US 6,147,109, US 6,150,500 and US 6,180,597 B1 give an overview of the knowledge of the possible activity of NO in the atherogenic process, pulmonary hypertension and stroke, as well as other conditions. The disclosure of these publications is incorporated herein by reference.

In US 6,147,109 it is disclosed that HMG-CoA reductase inhibitors may be used to increase the endothelial cell activity of NOS. US 6,150,500 discloses the regulatory peptides for NOS and discloses the use of these peptides in methods of modulating the activity of NOS enzymes. US 6,180,597 B1 describes the use of Rho GTPase function inhibitors for the same purpose.

We find, as discussed in the examples below, that Rg_1 appears to increase eNOS activity, both by increasing expression and by increasing the activity of the NOS enzymes.

Thus according to a further aspect of the invention we provide a method of treating a subject to increase endothelial cell nitric oxide synthase activity by increasing endothelial cell nitric oxide synthese expression in a tissue, comprising administering to the subject a panaxatriol, preferably the ginsenoside Rg_1 or a functionally equivalent derivative thereof. The invention also provides use of a panaxatriol, preferably the ginsenoside Rg_1 or a functionally-equivalent derivative thereof in the manufacture of a medicament for the treatment of a subject having a condition requiring increase of endothelial cell nitric oxide synthase activity by increasing endothelial cell nitric oxide synthase expression in a tissue.

As in the first aspect of the invention, other panaxatriols or mixtures thereof, as defined above, may be used in the same way but Rg_1 is preferred.

As in the first aspect of the invention, other panaxatriols may be used provided they have equivalent functionality, ie, any derivatisation does not significantly reduce the effect of increasing expression of eNOS. Generally functionally-equivalent derivatives have the same or a greater effect than the panaxatriol on increasing eNOS activity. If it is a lesser effect it is generally not less than 70%, usually not less than 80%, of the effect of the panaxatriol.

In this aspect of the invention activity of eNOS is increased by increasing the amount of eNOS in the tissue (increasing expression). It may additionally increase the activity of the enzyme itself.

Measurement of eNOS activity may be by any suitable manner known to the person skilled in the art. Suitable methods are described in US 6,147,109.

In this aspect of the invention the panaxatriol or functional equivalent is administered in amount sufficient to increase eNOS activity. This may involve increasing eNOS levels in endothelial cells above normal baseline level.

Dosages may be chosen by those skilled in the art according to the subject and the condition. Suitable amounts are from 2.5 ng to 500 μg panaxatriol per kg per day, preferably from 5 μg to 100 μg panaxatriol per kg per day. Treatment may be carried out for any period of time and may be over a period of months or even years. However, generally treatments will be carried out for from 1 to 60 days, preferably 1 to 30 days.

The invention may be applied to a subject, usually a mammal, preferably a human, having a variety of conditions in which increasing eNOS activity levels is advantageous. These include subjects having abnormally elevated risk of myocardial infarction or having experienced myocardial

infarction; subjects having abnormally elevated risk of pulmonary hypertension or having pulmonary hypertension; subjects having abnormally elevated risk of an ischaemic stroke or having experienced an ischaemic stroke; subjects suffering from impotence, kidney hypertension insulin deficiency, progressive renal disorder, gastric or oesophageal motility disorder; or a subject chronically exposed to hypoxic conditions. The low level of eNOS activity may be hypoxia-induced, chemically-induced or cytokine-induced.

As can be seen from the preceding paragraph, this aspect of the invention may be applied prophylactically or acutely.

In this aspect of the invention the Rg_1 or other panaxatriol may be administered in the form of a pharmaceutical composition comprising other actives which increase eNOS activity. Alternatively, such other actives may be administered separately. Alternatively, the Rg_1 may be the single pharmaceutical active.

In this aspect of the invention, as in the first aspect of the invention, the Rg_1 or other panaxatiol may be obtained by purification from ginseng and used as the racemate or as a single enantiomer or a non-racemic mixture of enantiomers (enantiomers referring to the C_{20} stereochemistry). It may be used in the form of a pharmaceutical composition formed by combining it with a pharmaceutically acceptable excipient.

This discovery is particularly surprising in view of the fact that it has been reported that ginsenosides act to inhibit iNOS (Radomski *et al*, 1990 "Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells", Proc. Natl. Acad. Sci. USA, 87:10043-10047; Park *et al*, 1996 "Ginsenoside-Rh_1 and Rh_2 inhibit the induction of nitric oxide synthases in murine peritoneal macrophages", Biochem. Mol. Biol. Int., 40:751-757; Yang *et al*, 1998 "Glucocorticoid inhibition of adjacent arthritis synovial

macrophage nitric oxide production: role of lipocortin 1". Clin. Exp. Immunol. 111:117-122; Miller *et al* 1996 "Progesterone inhibits inducible nitricoxide synthase gene expression and nitric oxide production in murine macrophages", J. Leukocyte Biol. 59:442-450; Lopez-Figueroa *et al*, 1998 "Regulation of nitric oxide synthase messenger RNA expression in the rat hippocampus by glucocorticoids, Nneuroscience, 87:439-446).]

Examples

Example 1

We tested angiogenic effects by means of an *in* vitro assay similar to that described in Lauder *et al*, Angiogenesis 1998;2;67-80. In this assay, "wounded" human umbilical vein endothelial cells (HUVEC) are exposed to increasing concentrations of Rg_1. In their normal physiological state endothelial cells remain quiescent but proliferate in injured tissue, causing angiogenesis. In mimicry, human umbilical vein endothelial cells were allowed to reach a confluent state in a well containing a semi-circular coverslip following which a "wound" to the monolayer was imparted by removing the cover slip, and leaving an area denuded of cells. In this system Rg_1 induced a concentration-dependent proliferation of serum starved HUVECs (EC_{50}=26.91nM). The proliferative effect appeared to be endothelial cell-specific as Rat-1 fibroblasts were insensitive to Rg_1 at the concentrations used.

Example 2

We also carried out *in vivo* tests using Rg_1. For these tests we used the sponge granuloma model similar to that described in Hu *et al* in Lab. Invest. (1995) 72, 601-610.

In this test, a sterile polyether sponge (170 mm^3) was implanted subcutaneously in Balb/c mice, and was injected with vehicle (0.1% EtOH in PBS) for 10 days. Angiogenesis was observed as the in-growth of blood vessels into the implant on day 15 (Figure 2a). Rg_1 (0.5

micrograms/sponge/day for 10 days) increased the neovascularisation with a mean vessel count of 30± 2.0 (Figure 2b), as also did treatment with 10 micrograms/sponge of Rg_1 (mean vessel count 31± 2) (Figure 2c).

Example 3

Results of experiments on the role of NO are shown in Figures 2d to 2h. Rg_1 produced a concentration dependent proliferation of HUVECS, following a 48 hour incubation after "wounding". We cultured HUVEC exposed to a maximal concentration of Rg_1 (125nM) in presence of a NOS inhibitor, Nώ-nitro-L-arginine methyl ester (L-NAME). In a concentration-dependent manner, L-NAME blocked the Rg_1-induced cell proliferation (*$p<0.01$ vs vehicle-treatment; + $p<0.01$ vs Rg_1 (125nM)-treatment (Figure 2d). Reversal of the inhibitory effect of L-NAME (10^{-5}M) was attained using increasing concentrations of Rg_1 or the NOS-substrate L-arginine. (#$p<0.01$ vs L-NAME (10^{-5}M) + Rg_1(125nM) - treatment group) (Figure 2e), suggesting the role of NO in Rg_1-induced angiogenesis. The block by L-NAME could also be overcome with higher concentrations of Rg_1 (Figure 2e), which indicated that Rg_1 might be increasing the synthesis of NOS.

We then tested whether NOS mediates the angiogenic response of Rg_1 in the *in vivo* sponge model. Coadministration of Rg_1 (10μg/sponge) with L-NAME (10μg/sponge) for 10 days blocked the angiogenic-effect of Rg_1. The effect of Rg_1 was similar to that of the angiogenic hepatocyte growth factor (HGF). The vessel counts in L-NAME treated group was similar to that of the vehicle-treated group. L-NAME did not affect the baseline angiogenic response. (@$p<0.01$ vs Rg_1-treatment) (Figure 2f). This suggested that NO might be modulating the Rg_1-induced angiogenesis in this model.

Further experiments with a selective e-NOS inhibitor, N^5-(1-iminoethyl)-L-ornithine (L-NIO) and an i-NOS inhibitor, S-methylisothiourea hemisulfate (SMT), suggested

the involvement of eNOS but not iNOS in Rg_1-induced angiogenesis. L-NIO produced a concentration dependent inhibition of Rg_1-induced HUVEC proliferation while SMT did not have any effect (+$p<0.01$ vs Rg_1 (125nM)-treatment (Figure 2g). The effects of L-NIO could be reversed using L-Arg or increasing concentrations of Rg_1. Subsequent Western analysis of HUVEC-lysates, fractionated on a 10% SDS-PAGE gel, revealed that as compared with the vehicle-treated cells, Rg_1 induced an expression of eNOS in HUVECs following 24 and 48 hours incubation (Figure 2h). We could not detect any iNOS in this system.

Example 4

In this experiment the effect of Rg_1 on tube formation by HUVECs and modulation by L-NAME was assessed. Cells were plated on extra-cellular matrix extracted from EHS murine sarcoma, and cultured for 16 hours in absence or presence of the relevant ginsenoside. Where the NOS-inhibitor, L-NAME, was used, the cells were pre-incubated with L-NAME, which was maintained throughout the entire duration of the experiment. Endothelial cells incubated on extracellular matrix align and form tubular structures as seen in the representative photomicrographs in Figures 3A to 3D. In this assay Figure 3A shows vehicle treated control. Figure 3B shows pretreatment with Rg_1 (125nM). This induced a concentration-dependent formation of tubes, which was quantified by counting the branches formed per view field. Figure 3C shows the results of treatment with L-NAME (10^{-3}M). This did not alter the basal tube formation. Figure 3D shows the results of pre-treatment of the cell with L-NAME followed by treatment with Rg_1 (125mM). The tube formation in this case was inhibited.

Concentration response of Rg_1 inducing angiogenesis is shown in Figure 3E and 3F.

Example 5

Gene expression profiling of Human Umbilical Vein Endothelial Cells induced by Rgl

Materials and method

Endothelial cells culture and drug treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics and maintained in M199 medium supplemented with 30μg/ml Endothelial Cell Growth Supplement (ECGS), 20% heat inactivated foetal calf serum (FCS), 1% penicillin - streptomycin, 50ng/ml amphotericin-B and 90μg/ml heparin. HUVECs at passage 6 were used in this study.

HUVECs ($2x10^5$/flask) were seeded in 0.1% gelatin coated T-75 culture flasks containing the full culturing medium. The HUVECs were allowed to reach 90% confluence in 48 hours then the medium was replaced with M199 medium supplemented with 1% FCS and 30ng/μg ECGS. After starving the cells for 24 hours, HUVECs were induced with 150mM Rg1 in M199 medium with 1% FCS and 3Ong/μl ECGS. After incubation for another 24 hours, the HUVECs were washed with phosphate buffered saline and harvested by treating with 0.05% (w/v) trypsin/0.02 (w/v) EDTA solution.

RNA extraction and labeling

Total RNA was extracted from the control and treatment groups using TRIZOL reagent (Life Technologies). Fluorescent labeling of the total RNA was performed using the direct incorporation method. Briefly, 50μg of total RNA as the starting materials was labeled in three separate labeling reactions. Each reaction includes total RNA (17μg), 4μl oligo(dT) primer (0.5μg/μl) (18-20mer; Life Technologies), 2μl RNase inhibitor (40U/μl) (Life Technologies) and 10μl DEPC-treated distilled water were added to a total volume of 20μl. The reaction mixtures were incubated at 70^0C for 10 minutes and chilled on ice for 2 minutes. After that, the mixtures were gently spun down, 8μl of 5X first-strand buffer, 4μl of O.IM DTT, 2μl of superscript II reverse transcriptase (200U/μl) (Life Technologies), 4μl of low-T dNTP mix (5mM dATP/dCTP/dGTP and 1mM dTTP; Amersham Pharmacia) and 3μl of 1mM Cy5-dUTP (for labeling control group) and Cy3-dUTP (for labeling treatment group) were added. The labeling mixture was

incubated at 42°C water bath for 2 hours and stopped by adding 5μl 0.5M EDTA and 10μl 1N NaOH followed by heating at 70°C heat block for 10 minutes. Finally, 25μl of Tris-HCi was added to neutralize the probes.

Probes purification

Cy3 and Cy5 labeled probes were washed separately using the Microcon-YM30 microconcentrator (Amicon). Probes were centrifuged at 12,000 rcf for 10 minutes in the present of TE buffer. The washing step was repeated by the addition of TE. The purified probe was collected by inverting the Microcon to a fresh tube and spun at 1,000 rcf for 3 min. Finally, both labeled probes were mixed and blocking cocktail consisted of 1μl of Poly dA (8mg/ml; Amersham Pharmacia), 1μl of Yeast tRNA (4mg/ml; Life Technologies) and 10μl of human Cot-1 DNA (1mg/ml, Life Technologies) were added. Then the probes were spun dry at 45°C to a final volume of about 20μl.

Hybridization and washing

The probes were heat denatured at 96°C for 5 minutes following the addition of 20μl 6X SSC, 0.2% SDS buffer. The denatured probes were chilled on ice for 3 minutes and gently spun down. 20μl of probe mixture was then added onto the array (OpArray Human Collage; Operon) and covered by the plastic coverslip (Sigma). The glass array was fitted into the hybridization cassette (ArrayIt), 40μl of SSC buffer was added into the small chamber inside the cassette before tightening the sealing screws. Hybridization was carried at 67°C water bath overnight. After the hybridization, the hybridized glass array was washed sequentially in 1X SSC and 0.03%SDS, 0.2X SSC and finally in 0.05X SSC for 8 minutes at room temperature. The slide was dried by centrifugation at 1,000 rcf before scanning.

Scanning and data analysis

Detection of the fluorescence signals was performed on a ScanArray5000 unit (GSI Lumonics) and analyzed with the QuantArray software. Normalization of data was done by the Microsoft Excel software.

Results

Scatter plot of hybridization intensities

Following image processing, the relative fluorescent intensities of the two scanned channels were normalized by setting the ratio of the house keeping genes (120 spots in this chip) to 1.0 (averaged Cy3 intensities/averaged Cy5 intensities = 1.0). This process would produce a normalization factor. Then the treatment group data was modified by multiplying this factor. Genes with equal expression levels in both groups will fall on the diagonal line while the differentially expressed genes will fall off the diagonal in the Scatter Plot (Figure 4).

Differentially expressed genes in HUVEC's due to Rgl

Those genes with the Cy3/Cy5 ratio greater than 1.5 will be considered as the up regulated genes, under the induction of Rgl (Figure 5).

The list of up-regulated genes (at least 1.5 fold) after the treatment of 15OnM Rgl

Gene names	Average of fold change	Standard deviation
P100	1.53	0.09
LAMB1	1.55	0.08
PAC1	1.56	0.07
LAMA4	1.61	0.05
RhoC	1.62	0.07
Amyloid beta precursor	1.65	0.08
RhoB	1.68	0.17
IQGAP1	1.86	0.17

Discussion

The effect of Rgl on HUVEC was analyzed using the state-of-the-art cDNA microarray technology. The HUVEC was synchronized by culturing in a starved medium (1% Fetal calf serum) for 24 hr. Then Rgl (150 nM) was added to the treatment group while the control would be treated the same

way except that Rgl was omitted. After incubation for 24 hr, the RNA from each group was extracted and labeled with either Cy3 or Cy5 for hybridization to the OpArray (human DNA microarray from Operon Technologies Inc. The Operon's Human Collage Array contains 70-mer oligos representing 320 genes from eight functional categories:
apoptosis, cancer, cell cycle, transcription factors, neuro/axon guidance, heat shock/stress, blood/inflammation, and aging. Each gene was printed in triplicate. The result of the analysis indicated that several genes were up-regulated by the action of Rgl on HUVEC. They include:

IQGAP1	Ras GTPase-activating-like protein
RhoB	RhoB
RhoC	Ras homolog gene family, member C
Rho	Ras homolog gene family, member A
AbetaPP	Amyloid beta precursor protein
Rac-alpha	Rac protein kinase alpha
Racl	Rho family, small GTP binding protein Racl
PRAD1	Cyclin D1
HSP 90	heat shock protein 90 kDa
TGFbeta3	Transforming growth factor beta 3
CALM2	Calmodulin
LAMA4	Laminin alpha 4
LAMB1	Laminin beta 1
LAMB2	Laminin beta 2

The Rho, RhoB, RhoC and Racl are members of the Rho family of GTPases which belong to the Ras superfamily of small GTPases. IQGAP1 is most up-regulated in our system.

IQGAP1 interacts with cadherins and catenins has been implicated in the regulation of cadherin mediated cell-cell adhesion. Besides, IQGAP1 is found to interact with Ca^{2+}, calmodulin and F-actin. Thus, IQGAP1 seems to act as a molecular link between several signaling pathways to mediate cell-cell adhesion which is critical to the tube formation of HUVEC.

Laminins are basement membrane glycoproteins that promote cell adhesion, migration differentiation and

growth. Laminin-derived peptides have been shown to induce single endothelial cells to attain ring-like structures surrounding a hollow lumen resembling the capillaries.

TGF beta-1(an isoform of TGF beta-3) has been reported to induce endothelial cell separation by initiating actin contraction while maintaining the adherens junction complex as demonstrated by the presence of catenins and VE-cadherin at the cell periphery. Since VE-cadherin has been implicated in the adherens junctions for tube formation, TGFs may play an important role in rearrangment of the actin fibers during cell migration and tube formation.

CLAIMS

1. Use of a panaxatriol without panaxadiol in the manufacture of a medicament for the treatment of a condition requiring stimulation of angiogenesis.

2. A use according to claim 1 wherein the panaxatriol is a naturally-occurring ginsenoside, preferably Rg_1.

3. A use according to claim 1 or claim 2 in which the condition is selected from the group consisting of non-union fracture, hair loss, gangrene, treatment after transplantation, treatment after myocardial infarction, prevention of myocardial infarction, treatment after ischaemic stroke, prevention of ischaemic stroke, transplantation, and in neurological and ophthalmological conditions where enhanced neovascularisation is deemed to be beneficial, ulcer healing and wound healing.

4. A use according to any preceding claim in which the panaxatriol has been obtained by purification from ginseng.

5. Use of a pharmaceutical composition in the manufacture of a medicament for use in a method for the treatment of a condition requiring stimulation of angiogenesis, wherein the composition has been prepared by (i) providing ginseng or a ginseng extract, (ii) obtaining from the ginseng or ginseng extract a panaxatriol in purified form, optionally (iii) derivatising the panaxatriol, and (iv) combining the purified and optionally derivatised panaxtriol with a pharmaceutically acceptable excipient.

6. A use according to claim 5 in which the pharmaceutical composition comprises at least one component which is not naturally present in ginseng.

7. A use according to any preceding claim in which panaxatriols are the only pharmaceutical actives.

8. A use according to claim 7 in which the ginsenoside Rg_1 is the sole pharmaceutical active.

9. A use according to any preceding claim in which the panaxatriol is applied to the subject to be treated encapsulated in liposomes or in an implant.

10. A use according to claim 9 wherein the treatment comprises injecting liposomes into the bloodstream or application of an implant into the surface of a wound or application of an implant to the surface of an ulcer.

11. A pharmaceutical composition which comprises a panaxatriol encapsulated in liposomes, microsomes, microsponges or a controlled drug release system

12. A pharmaceutical composition which comprises a panaxatriol in an implant made of natural, synthetic or semi-synthetic material, preferably a collagen implant.

13. A pharmaceutical composition which comprises a panaxatriol impregnated in a polymeric device suitable for implantation in the human body.

14. A pharmaceutical composition according to any of claims 11 to 13 in which the panaxatriol is Rg_1.

15. A method of testing a target compound for efficacy in promoting angiogenesis comprising performing an assay which tests whether the target compound acts to upregulate any gene in the group consisting of IQGAP1, RhoB, RhoC, Rho, Abeta PP, Rac-alpha, Rac1, PRAD1, HSP90, TGFbeta3, CALM2, LAMA4, LAMB1, LAMB2.

16. A method according to claim 15 in which the gene is selected from the group consisting of IQGAP1, RhoB, RhoC, Rho, AbetaPP, Rac-alpha, Rac1, HSP90 and CALM2.

17. Use of a panaxatriol without panaxadiol in the manufacture of a medicament for the treatment of a condition requiring increased endothelial cell nitric oxide synthase expression.

18. Use according to claim 17 wherein the panaxatriol is a naturally occurring ginsenoside, preferably Rg_1.

19. A method of treating a human or other animal subject comprising stimulating angiogenesis by using a panaxatriol without panaxadiol.

20. A method of treating a human or other animal subject comprising stimulating angiogenesis by using the panaxatriol ginsenoside Rg_1.

21. A method of treating a human or other animal subject by stimulating angiogenesis using a pharmaceutical composition, wherein the composition has been provided by providing ginseng or a ginseng extract, obtaining from the ginseng or ginseng extract a panaxatriol in purified form, optionally derivatising the panaxatriol, and combining the purified and optionally derivatised panaxatriol with a pharmaceutically acceptable excipient.

Fig.1(a).

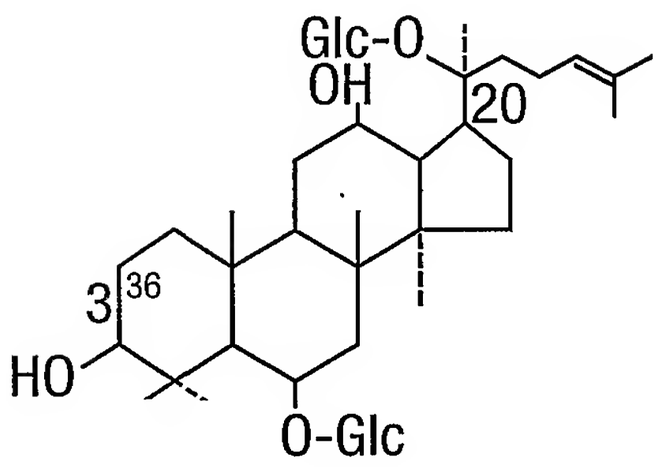


Fig.1(b).

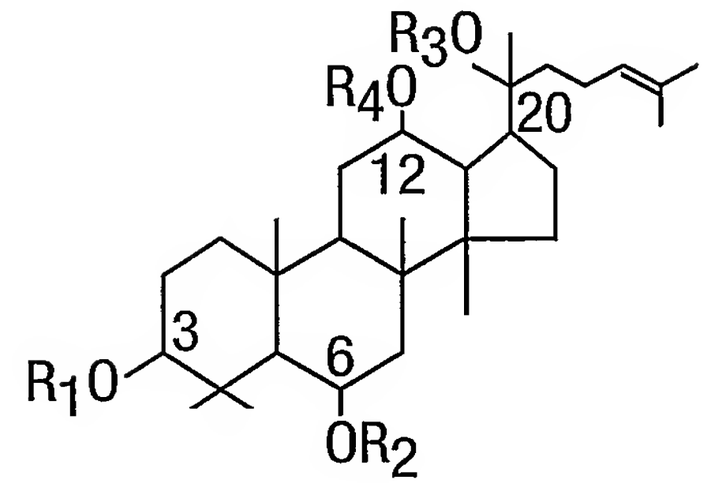
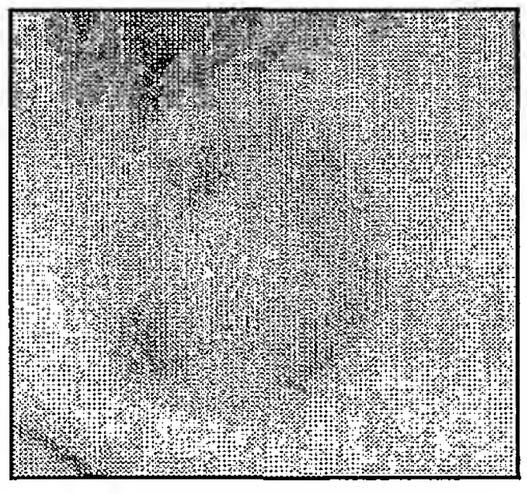


Fig.2(a).

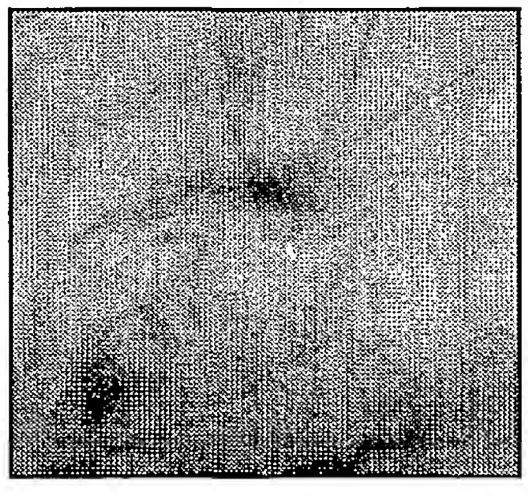


Vehicle control

Fig.2(b).

Rg_1 (0.5 µg/sponge)

Fig.2(c).



Rg_1 (10 µg/sponge)

Fig.2(d).

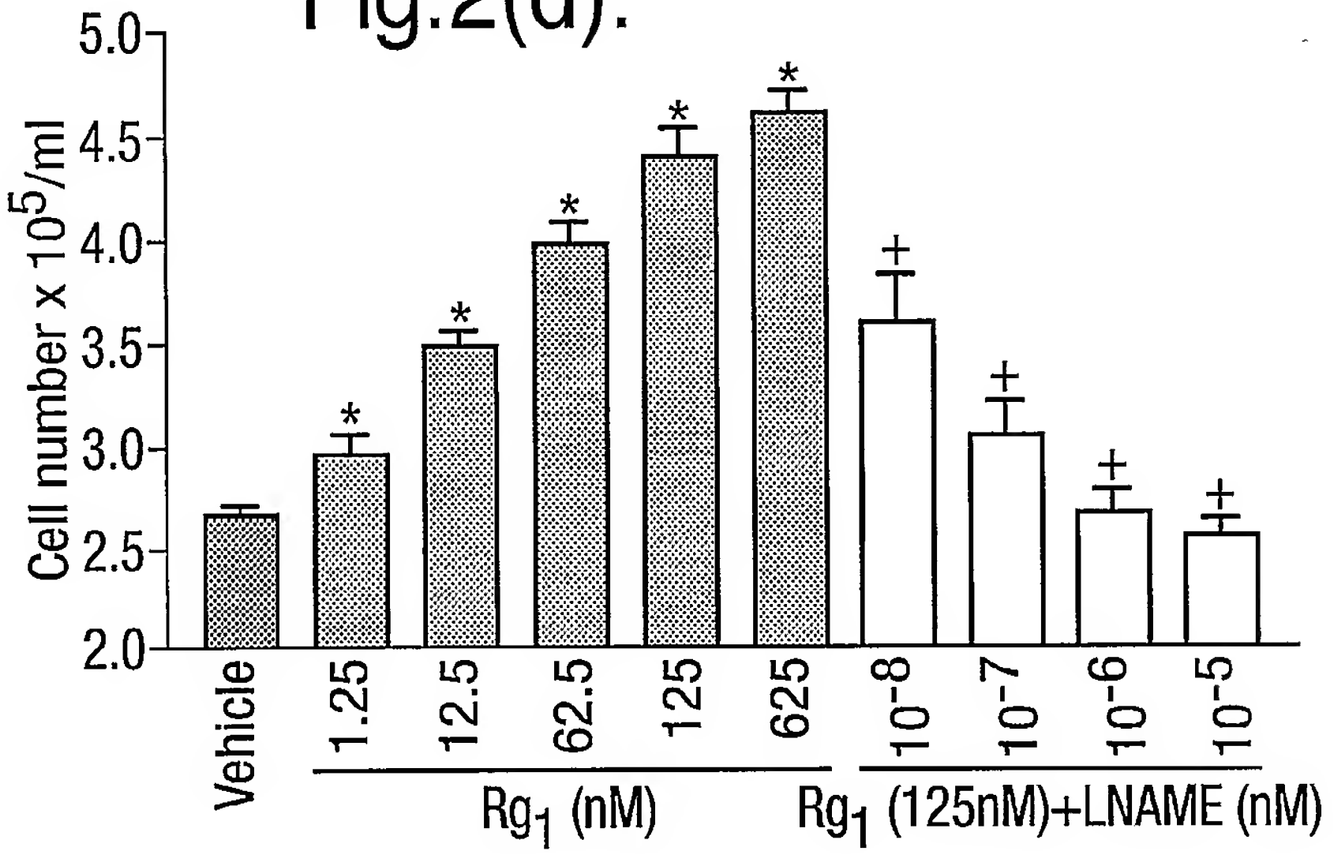


Fig.2(e).

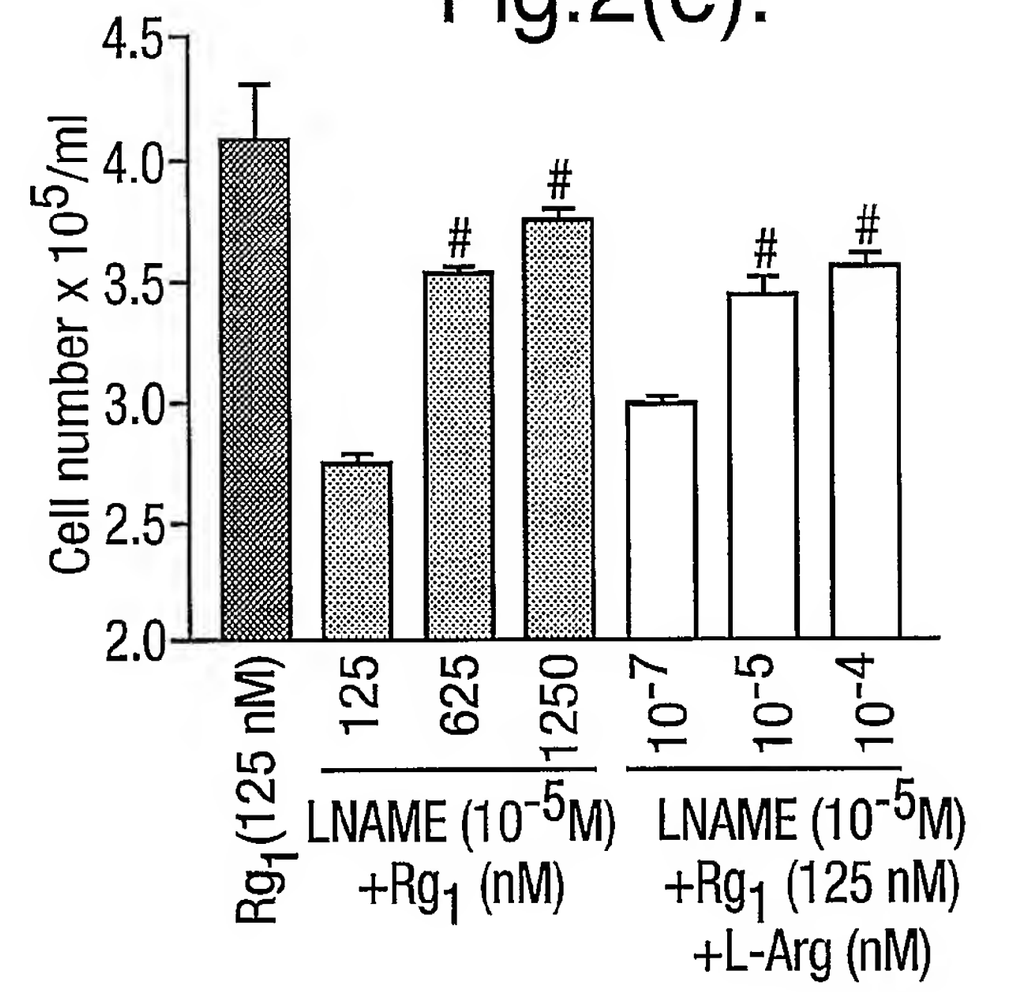


Fig.2(f).

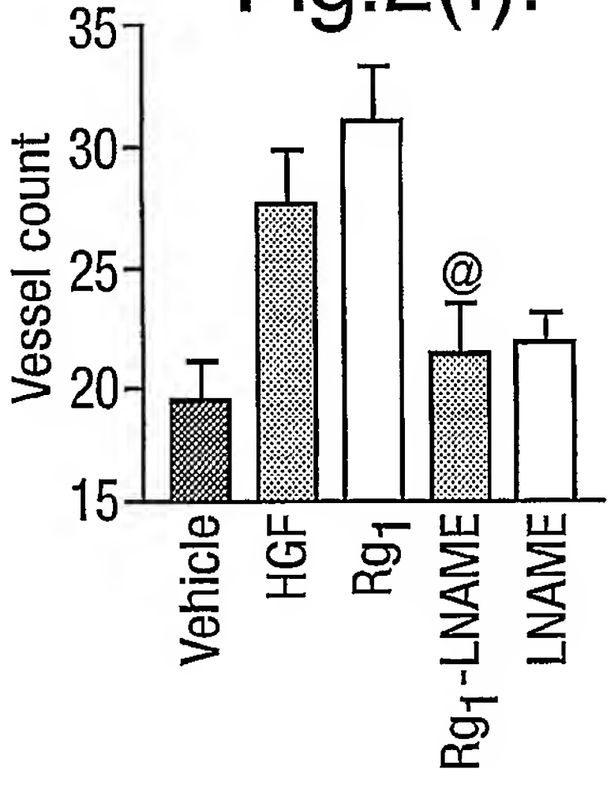
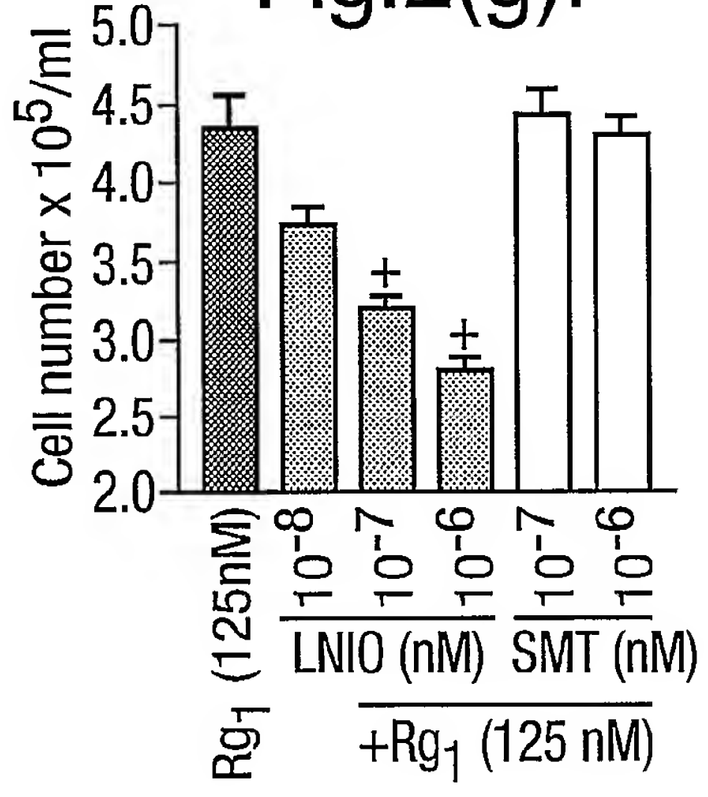


Fig.2(g).



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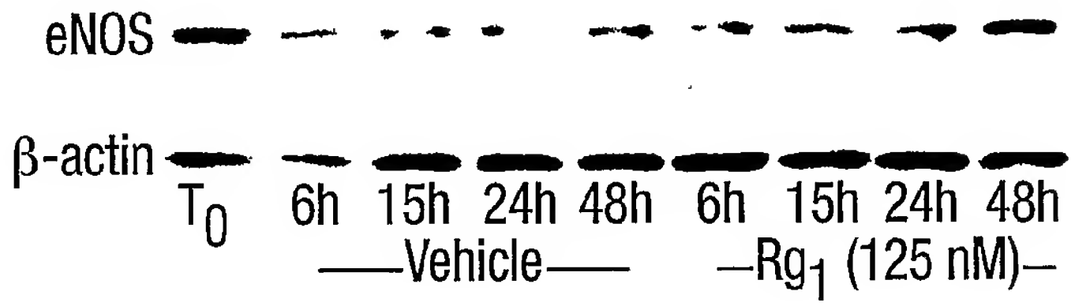


Fig.3(a).

Control

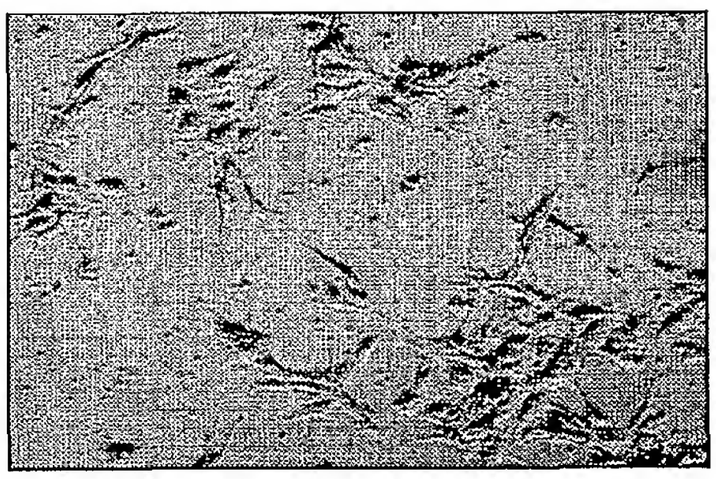


Fig.3(c).

L-NAME [10^{-3} M]

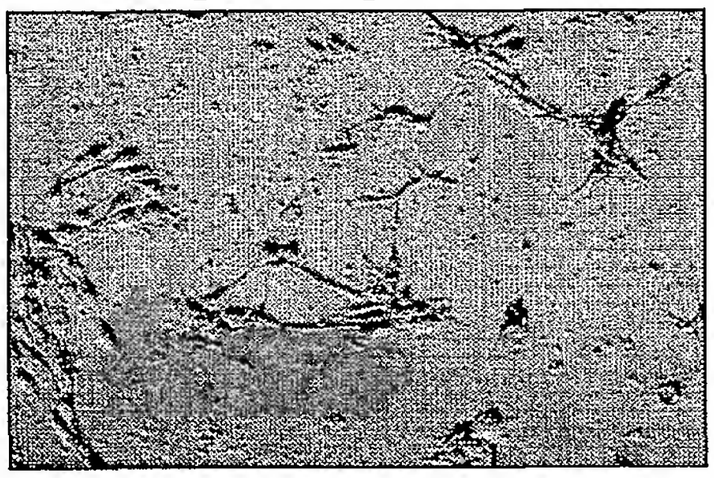


Fig.3(b).

Rg_1 [125 nM]

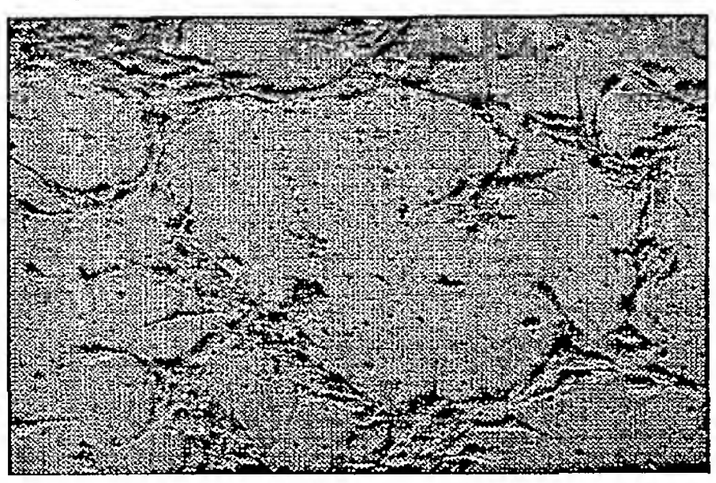


Fig.3(d).

Rg_1 [125 nM] +
LNAME [10^{-3} M]

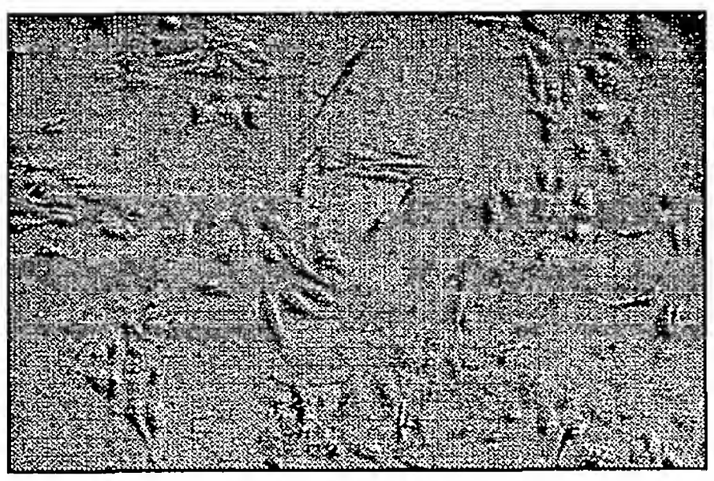


Fig.3(e).

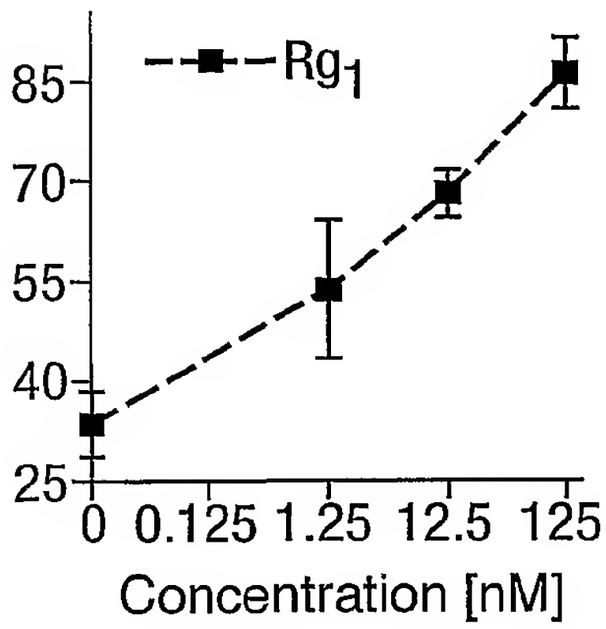


Fig.3(f).

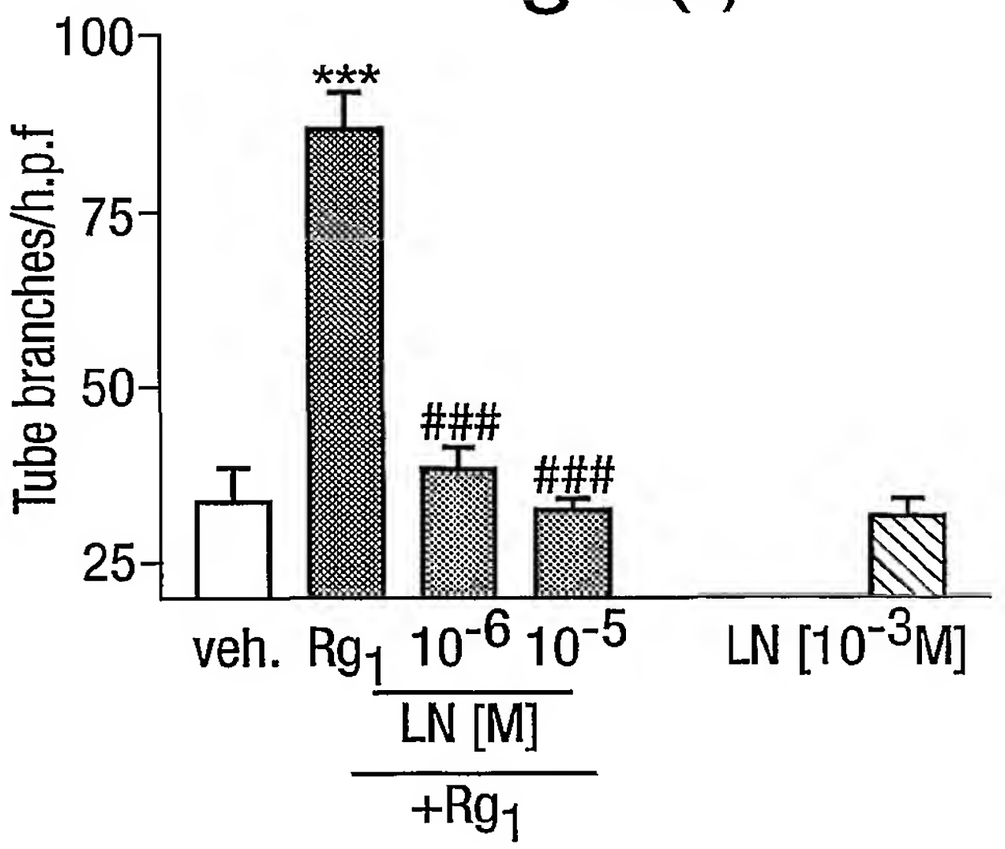
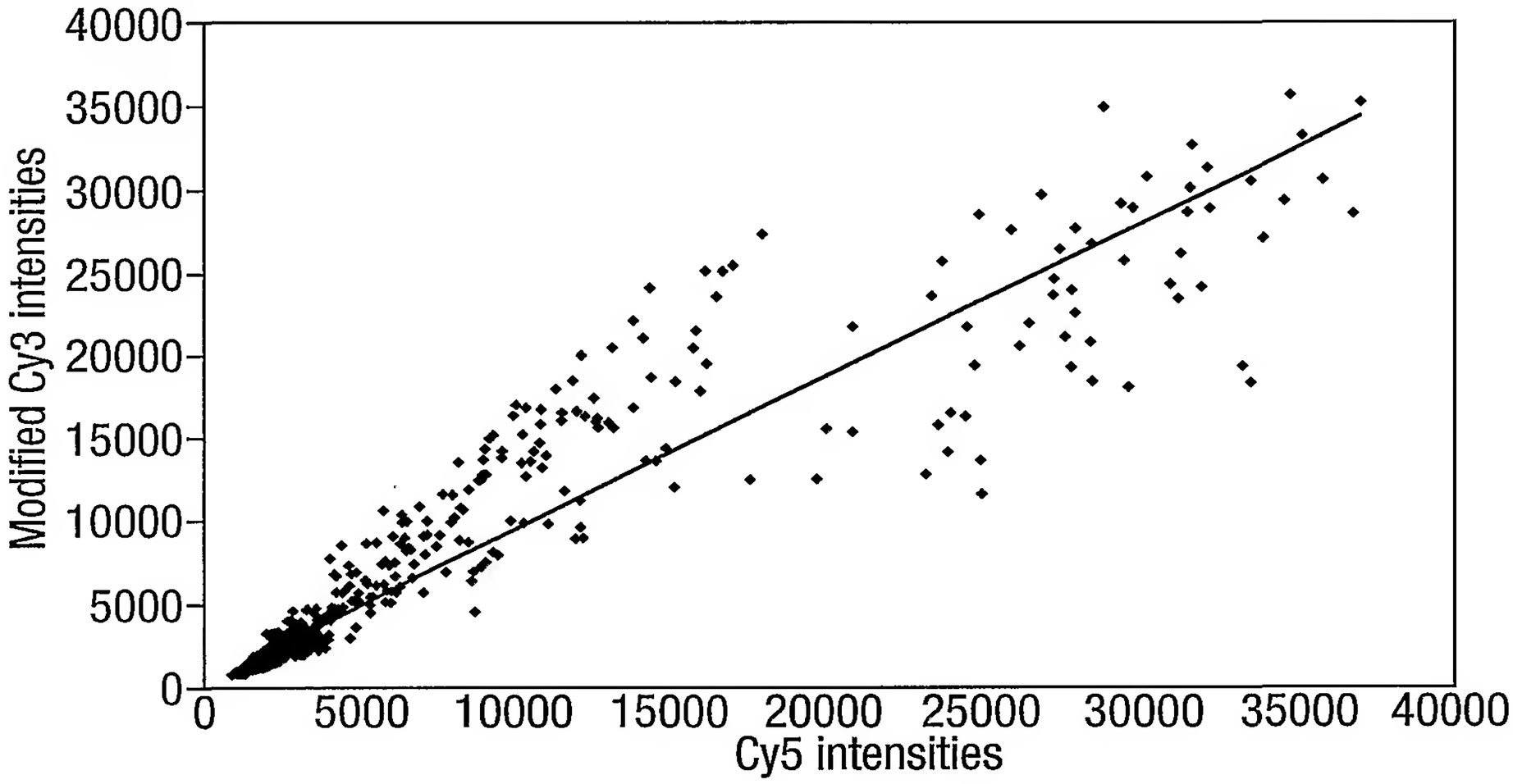


Fig.4.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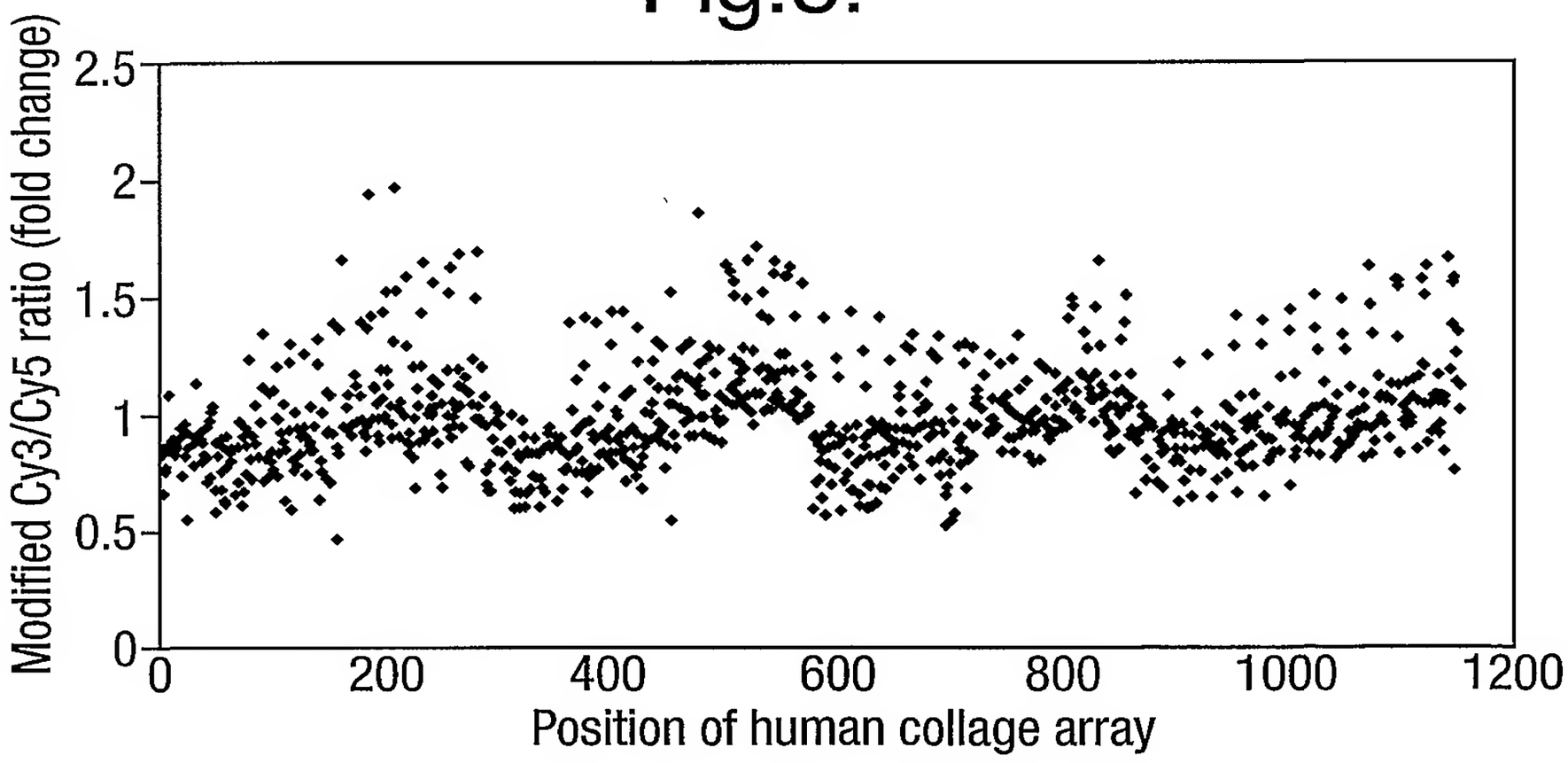
30000
25000
20000
15000
10000
5000
0
0
5000
10000
15000
20000
25000
30000
35000
40000
Cy5 intensities

Fig.5.


Modified Cy3/Cy5 ratio (fold change)
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2
1.5
1
0.5
0
0
200
400
600
800
1000
1200
Position of human collage array